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Some Biological Effects of Carbamoyloxyurea, an Oxidation Product of Hydroxyurea

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Carbamoyloxyurea, an oxidation product of hydroxyurea, is bactericidal for *Escherichia coli*. Drug-induced killing is independent of cellular metabolism; ribonucleic acid and protein syntheses are the processes most affected, and the lethal action is accompanied by degradation of cellular deoxyribonucleic acid. In all of these effects the drug differs from hydroxyurea, a primarily bacteriostatic agent that inhibits deoxyribonucleic acid synthesis, whose lethal action ultimately depends on cellular activity.

Recent studies on the mode of action of hydroxyurea have led to the realization that the action of this drug on bacteria could be divided into two phases: (i) an immediate and reversible inhibition of deoxyribonucleic acid (DNA) synthesis and (ii) a delayed effect characterized by cellular death and modification of the cellular DNA (8, 11, 14, 16). This secondary action of hydroxyurea was shown to be dependent on energy metabolism (11). In vitro studies have indicated that, whereas fresh solutions of hydroxyurea were without effect on purified DNA, aged hydroxyurea solutions were capable of causing alterations of the DNA structure (15; S. J. Jacobs, Ph.D. Dissertation, Columbia University, 1968). Such aged hydroxyurea preparations have been shown to contain isohydroxyurea, $H_2N—C(=O)—ONH_2$ (17), *N*-carbamoyloxyurea, $H_2N—C(=O)—N(—H)—O—C(=O)—NH_2$ (Jacobs and Rosenkranz, *in press*), and an unidentified nitrosourea (9, 17). Recent studies (9; Jacobs and Rosenkranz, *in press*) indicated that the in vitro degradation of DNA by aged hydroxyurea preparations was caused by carbamoyloxyurea, an oxidation product of hydroxyurea (1, 6, 7).

The present report is concerned with the effects of carbamoyloxyurea on *Escherichia coli*, and the possibility that the delayed effect of hydroxyurea could be caused by carbamoyloxyurea.

MATERIALS AND METHODS

Bacterial strains and media. The properties of *E. coli* C600, the hydroxyurea-resistant mutant *E. coli* C600/HU, the isohydroxyurea-resistant mutant *E. coli* C600/isoHU, and the composition of the liquid medium HA, have been described (12, 13, 18).

For determining the number of viable bacteria, serial dilutions (0.1 ml) of cell cultures were plated on Columbia-base agar (3).

Metabolic techniques: All the techniques used in this study have been described previously (14, 16). Whenever radioactive thymidine was used, the medium was supplemented with uridine (366 μg/ml) to inhibit and repress thymidine phosphorylase (2).

Materials. *N*-carbamoyloxyurea (*N,O*-dicarbamoylhydroxylamine, SQ 10,726) was generously donated by B. Stearns, Squibb Institute for Medical Research. Only freshly prepared solutions of this compound were used. Azauracil and ^3H-labeled compounds were purchased from Schwarz Bio-Research, Inc; ^{14}C-thymidine from New England Nuclear Corp.; chloramphenicol from Parke, Davis & Co.; and sodium azide from Fisher Scientific Co.

RESULTS AND DISCUSSION

Exposure of bacteria to carbamoyloxyurea levels in excess of 0.005 molar resulted in cellular death and an inhibition of the increase in turbidity of treated cultures (Fig. 1). Hydroxyurea, on the other hand, is primarily bacteriostatic and allows increases in turbidity to occur, i.e., it induces unbalanced growth (14, 15). In further contrast to the effects of hydroxyurea which selectively blocks DNA synthesis, carbamoyloxyurea affects ribonucleic acid (RNA) and protein production primarily (Fig. 2). The bactericidal action of carbamoyloxyurea was not prevented by inhibitors of RNA [azauracil (5)], protein [chloramphenicol (4)], and DNA [hydroxyurea (14)] synthesis, or by sodium azide, which inhibits oxidative phosphorylation (Table 1). Conversely, the experimental results revealed that these blocking agents actually increased the lethal effect of carbamoyloxyurea. This is in direct contrast to the effects of metabolic in-

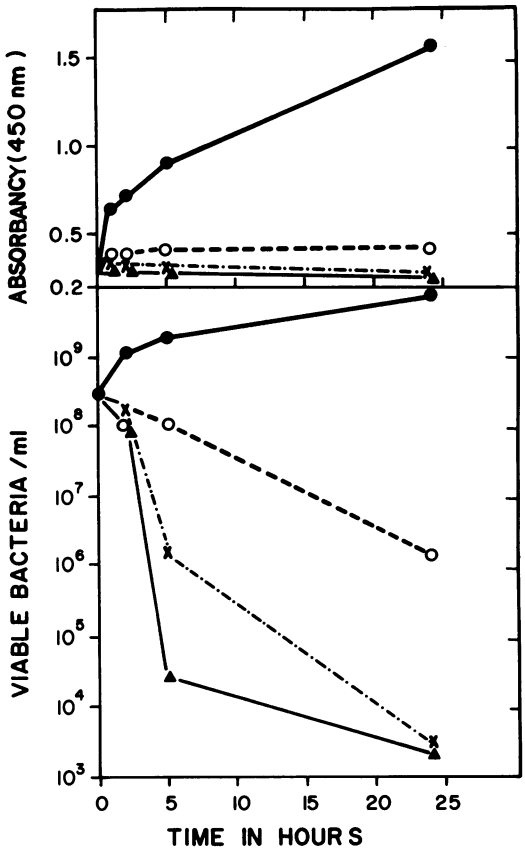

FIG. 1. *Effect of carbamoyloxyurea on the turbidity and viability of a culture of E. coli C600. Bacteria (E. coli C600) in medium HA (13) were brought to the exponential-growth phase, at which time they were distributed into flasks containing premeasured amounts of carbamoyloxyurea. The turbidity (absorbance measured at 450 nm, Bausch & Lomb Spectronic 20 colorimeter) and the number of viable bacteria were determined at intervals. Symbols: (●) control; (○) 0.01 M, (×) 0.05 M, and (▲) 0.1 M carbamoyloxyurea, respectively.*

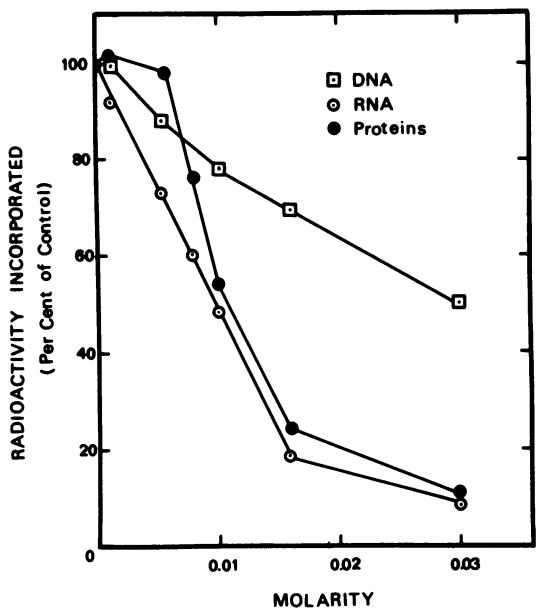

FIG. 2. *Effect of carbamoyloxyurea on the macromolecular metabolism of E. coli C600. Bacteria in medium HA were brought to the exponential-growth phase (approximately 1.5 × 10^8 cells/ml). Portions of the cultures were then distributed into flasks containing premeasured amounts of carbamoyloxyurea and of the radioactive precursor: ^{3}H-thymidine (7.4 × 10^{-6} M, 0.5 μc/ml) and non-labeled uridine (366 μg/ml) for DNA, uridine-5-^{3}H (2.0 × 10^{-4} M, 2.4 μc/ml) for RNA and ^{3}H-lysine (8.9 × 10^{-5} M, 0.87 μc/ml) for proteins. The cultures were incubated with aeration for 0.5 hr, and then duplicate 1-ml portions were removed from each culture for the determination of radioactivity incorporated into acid-insoluble form. The samples containing ^{3}H-thymidine and ^{3}H-lysine were digested with alkali and hot (90 C) acid, respectively (17). The radioactivity present in the cultures incubated in the absence of carbamoyloxyurea was 10,524, 65,576, and 32,221 counts per min per ml for ^{3}H-uridine, ^{3}H-thymidine, and ^{3}H-lysine, respectively. Symbols: ⊡, DNA; ⊙, RNA; and ●, proteins.*

hibitors on the bactericidal action of hydroxyurea (11).

Exposure of cells to bactericidal levels of the drug resulted in partial degradation of the cellular DNA to acid-soluble products. This was not accompanied either by a parallel depolymerization of the bacterial RNA or by lysis of the cells. The solubilization of the DNA was prevented by sodium azide (Table 2). It is of interest that the delay (5 hr) which preceded this depolymerization was identical to the one which preceded cellular death. Preincubation of the drug for 5 hr did not shorten this lag.

Although cells resistant to hydroxyurea and isohydroxyurea showed some cross-resistance to the lethal effects of carbamoyloxyurea (Table 3), the results do not allow us to conclude that carbamoyloxyurea is the principle responsible for the lethal action of these drugs.

The data suggest that a direct reaction between DNA and carbamoyloxyurea is not the basis of the lethal effect of hydroxyurea, even though such a reaction can be demonstrated in vitro (9). If carbamoyloxyurea were, in fact, the lethal principle of hydroxyurea, it should cause immediate death of treated bacteria. This, however, was not the case: killing was usually preceded by a period of bacteriostasis. It is of further interest that the lethal action of carbamoyloxyurea when

it occurred was accompanied by degradation of cellular DNA. However, although sodium azide magnified this killing, it prevented the depolymerization of the cellular DNA, thus suggesting that DNA degradation was not a corequisite for cellular death.

Carbamoyloxyurea is a potent antibacterial agent, and the cross-resistance studies suggest that its mode of action is related to that of hy-

TABLE 1. *Effect of metabolic inhibitors on the lethal action of carbamoyloxyurea*[a]

Additions[b]	Viable bacteria per ml		
	0 hr	5 hr	24 hr
None	2.3×10^8	3.1×10^9	4.8×10^9
COU		1.8×10^8	1.8×10^6
COU + CM		1.3×10^8	4.6×10^4
CM		2.9×10^8	2.3×10^8
COU + Azauracil		1.8×10^8	4.5×10^4
Azauracil		5.8×10^8	8.4×10^8
COU + HU		5.6×10^7	4.9×10^5
HU		2.0×10^8	7.3×10^7
COU + NaN_3		1.7×10^8	3.5×10^4
NaN_3		4.1×10^8	5.7×10^6

[a] Bacteria (*E. coli* C600) were brought to exponential growth phase and then distributed into flasks containing premeasured amounts of azauracil (300 μg/ml), CM (30 μg/ml), COU (0.01 M), HU (0.1 M), or NaN_3 (0.01 M).

[b] Abbreviations: CM, chloramphenicol; COU, *N*-carbamoyloxyurea; HU, hydroxyurea; NaN_3, sodium azide.

TABLE 3. *Effect of carbamoyloxyurea on the viability of several Escherichia coli strains*[a]

Molarity of carbamoyloxyurea	Time	Per cent survival		
		C600	C600/HU	C600/isoHU
	hr			
0	0	100	100	100
0	24	2,740	2,918	2,415
0.01	24	2.6×10^{-1}	5.5×10^{-1}	1.2
0.03	24	8×10^{-2}	3×10^{-1}	
0.05	24	1×10^{-3}	5×10^{-5}	
0.10	24	7×10^{-4}	2×10^{-6}	

[a] Bacteria in medium HA were brought to exponential-growth phase [*E. coli* C600, the parent strain, 2.7×10^8; *E. coli* C600/HU, the hydroxyurea-resistant strain, 2.2×10^8; *E. coli* C600/isoHU, the isohydroxyurea-resistant strain, 1.7×10^8]. The bacteria were then distributed into flasks containing premeasured amounts of carbamoyloxyurea. Viable bacteria were enumerated at intervals.

TABLE 2. *Carbamoyloxyurea and the stability of cellular constituents*[a]

Additions[b]	Viable bacteria per ml			Turbidity (450 nm)		Radioactivity retained[c]				
						Deoxyribonucleic acid			Ribonucleic acid	
	0 hr	5 hr	24 hr	0 hr	24 hr	0 hr	5 hr	24 hr	0 hr	24 hr
Expt I										
None	1.3×10^8	3.0×10^9	4.6×10^9	0.26	1.8	9,917	10,060	10,147		
0.01 M COU		1.4×10^8	4.7×10^6		0.44		9,056	6,874		
0.01 M COU + NaN_3		1.7×10^8	1.6×10^4		0.26		9,493	8,914		
0.01 M NaN_3		2.2×10^8	4.0×10^6		0.48		9,528	9,582		
Expt II										
None	1.3×10^8	2.4×10^9	5.4×10^9	0.25	1.8				23,761	26,326
0.01 M COU		1.0×10^8	5.1×10^5		0.39					27,517
0.01 M COU + NaN_3		9.5×10^7	1.0×10^4		0.25					25,402
0.01 M NaN_3		2.3×10^8	3.2×10^6		0.53					26,015

[a] Bacteria (*E. coli* C600) were brought to exponential growth phase in medium HA containing either ^{14}C-thymidine and 366 μg of uridine/ml (Experiment I) or 2×10^{-4} M ^3H-uridine (experiment II). When a bacterial density of approximately 1×10^8/ml was reached, the cells were washed, resuspended in the original volume of fresh prewarmed medium HA, and incubated for 30 min. Portions of the culture were then distributed into flasks containing premeasured amounts of COU and NaN_3. At intervals, quadruplicate 1.0-ml samples were removed for the determination of radioactivity retained.

[b] Abbreviations: COU, carbamoyloxyurea; NaN_3, sodium azide.

[c] Values expressed as counts per minute per milliliter.

droxyurea and isohydroxyurea; the nature of this relationship and the function of carbamoyloxyurea in the previously proposed (17, 18) scheme remain to be established.

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